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EXHIBIT B

9

The Utility of Fusion Proteins in the Development of Vaccines

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PRINCIPLES OF FUSION PROTEIN CONSTRUCTION

With the advent of recombinant DNA technology it has become possible to clone eukaryotic genes in microorganisms such as *E. coli*. It appeared, however, that the efficient expression of cloned eukaryotic genes in bacteria is not a trivial task, because bacteria often rapidly degrade foreign proteins (see Chapter 8), especially portions thereof. To date and despite the fact that protease-deficient *E. coli* mutants (e.g., lon mutants) have been constructed (1), no rules or principles have been established which could be used to predict if a given foreign antigen can be stably recovered after expression in *E. coli* or if it will be subject to proteolytic degradion. In this context it is interesting to note that *E. coli* has at least eight different proteases, of which five are strictly cytoplasmic and two are strictly periplasmic (2). The problem of instability was first encountered with eukaryotic proteins, such as rat and human insulins (3,4), rat growth hormone (5), and the peptide hormone somatostatin (6). Subsequently, it was found that the fusion of the coding sequence of the eukaryotic gene to the 3′ end of an *E. coli* gene resulted in the expression of a stable fusion protein (3-6). Not much later, it was observed that viral antigens could also be stably expressed after the fusion of the respective genes to *E. coli* proteins.

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In Recombinant DNA Vaccines by R. E. Isaacson Marcel Dekker, New York. 1992

Early examples of such experiments were the fusion of antigenic determinants of the hemagglutinin (HA) of human influenza virus (7) or of the hepatitis surface antigen (8,9) to the *E. coli* β-galactosidase (product of the *lacZ* gene), the fusion of hepatitis B surface antigen (HBsAg) (9,10) or the rabies virus 60-kDa glycoprotein (Rab-GP) (11) to the *E. coli* β-lactamase (product of the *ampC* gene), the fusion of the capsid protein VP1 of food-and-mouth-disease virus (FMDV) to the replicase of the bacteriophage MS2 (12) and of the capsid protein VP3 of the same virus (13), and of the HA of human influenza virus (14) to the *E. coli* trpLE' protein and of the vesicular stomatitis virus glycoprotein (VSV-GP) to the *E. coli* trpE protein (15).

The great potential of the fusion protein approach became evident in the report of Kleid et al. (13), in which it was shown for the first time that a fusion protein (the trp LE'::VP3 chimeric protein) was able to elicit high levels of neutralizing antibodies when injected into cattle and swine and, more importantly, that it was able to protect the animals against challenge infection with FMDV. In the report of Davis et al. (14) several trpLE'::influenza virus HA fusion proteins were constructed, expressing parts or the complete HA protein. Each of the fusion proteins were purified and used to immunize mice and rabbits. The antibody produced was shown to bind to the HA fusion proteins (as expected), but also to detergent-treated viral HA, to HA on intact virions, and to the HA expressed on the surface of influenza virus-infected cells. These results demonstrated that fusion proteins expressed in bacteria can elicit antibodies that recognize at least some determinants on the native viral protein.

In all these early examples attempts to express the respective viral genes directly, i.e., in an unfused form, resulted in no or only negligible expression rates which were too low to isolate sufficient amounts of the desired viral antigen. In contrast, after fusion of the viral antigen to the respective *E. coli* acceptor protein, between 0.05% (lacZ::HBsAg) (8) and 20% (trpLE'::HA) (14) of the total cellular protein constituted the respective fusion protein (Table 1). The results also demonstrated that the yield of fusion proteins

Table 1 Expression Levels of Various Fusion Proteins

Gene fusion	% of total cellular protein	Ref.
lacZ::HBsAg	0.05	8
trpE::VSV-GP	1	15
ampC::Rab-GP	3	11
lacZ::HA	5-7	7
ampC::HBsAg	8.5	10
trpLE'::FMDV-VP3	17	13
trpLE'::HA	10-20	14

using the same *E. coli* acceptor protein (for example, β-galactosidase) varied considerably, which meant that the nature of the foreign antigen contributed strongly to the extent of its stabilization. In contrast to all other above-mentioned viral antigens, the β-lactamase::HBsAg and β-galactosidase::HBsAg fusion proteins reacted poorly if at all with antibodies to the native surface antigen but were nevertheless able to induce specific antibody formation in rabbits, although at a low titer and only after booster injections (9). In this context it is important to remember that the dissociated HBsAg is much less immunogenic than the 22-nm HBsAg particle and that the result of poor reactivity of the fusion protein is not particularly surprising, since it is evident that the conformation of viral antigens fused to *E. coli* proteins will probably differ from its natural conformation in the viral particle.

A SHORT SURVEY OF WIDELY USED FUSION VECTOR SYSTEMS

Most fusion vector systems use highly expressed *E. coli* proteins or parts thereof as acceptors to which the antigen of interest will be fused. In the most simple situation, the foreign antigen is fused to the C-terminus of the *E. coli* protein (in the examples given above to the C-terminus of β-galactosidase, β-lactamase, or the *trp*E and *trp*LE' gene products). This method has the advantage that the prokaryotic 5′ region of the gene encoding the "acceptor" protein, including its natural promoter and ribosome binding sites, can be used without modifications, which can be quite complicated and time consuming. The first fusion vectors developed thus used naturally occurring unique restriction sites close to the 3′ end of the acceptor gene for the insertion of the foreign DNA fragment.

β-Galactosidase Fusion Vectors

Fusions to the C-Terminus of β-Galactosidase

In the case of the *lacZ* gene, the *Eco*RI cleavage site located 53 base pairs upstream of the β-galactosidase termination codon at a position corresponding to amino acid 1006 (16) was successfully used to express fusion proteins (4-8). The loss of enzymatic activity of β-galactosidase upon insertion of foreign DNA in the naturally occurring *Eco*RI site of the *lac*Z gene can be used to distinguish recombinant from nonrecombinant clones by their inability to produce blue colonies or plaques on X-gal indicator plates (negative screening). In order to provide for more cloning sites, newer generations of β-galactosidase fusion vectors carry synthetic DNA fragments as polylinker regions at a position corresponding to the C-terminal end of the almost full-length (17,18) or of truncated versions of the *lac*Z gene (19,20). Here, the unique restriction sites allow for the insertion of foreign DNA fragments in all three possible reading frames. In the plasmid vectors developed by

Ruether and Mueller-Hill (17), insertion of foreign DNA in the correct reading frame results in the synthesis of an enzymatically active β-galactosidase fusion protein, which can be easily detected in a *lacZ*-negative host strain. By using the pBD vectors of Broeker (19) or the pSEM vectors of Knapp et al. (20), very high yields (up to 40% of total cellular protein) of truncated β-galactosidase fusion proteins can be isolated. Because of the truncation of β-galactosidase (in the pSEM vectors, for example, to its N-terminal 375 amino acids), the molar ratio of the desired antigen to the *E. coli* acceptor protein is improved.

Fusions to the N-Terminus of β-Galactosidase

In a different type of β-galactosidase fusion vector, foreign DNA can be inserted into cloning sites present several codons after the natural β-galactosidase initiation codon (21-24). In this configuration, the foreign antigen will be expressed at the N-terminus of β-galactosidase, resulting in a fusion protein retaining its enzymatic β-galactosidase activity (positive screening). In Broekhuijsen et al. (23), two expression plasmids were constructed encoding VP1 cDNA sequences of FMDV. Substantial amounts of the two VP1::β-galactosidase fusion proteins were synthesized, containing either one (amino acid regions 140-160) or two (amino acid region 140-160 and 200-213) antigenic determinants of the virus. The fusion protein containing the amino acid region 140-160 of VP1 efficiently induced antibodies in rabbits specifically reacting with FMDV and was also capable of eliciting neutralizing antibodies. The fusion protein containing both antigenic determinants did not efficiently induce antibodies reacting with FMDV, probably due to improper three-dimensional folding of the fusion protein, which influences immunogenicity.

Open Reading Frame β-Galactosidase Fusion Vectors

A third type of β-galactosidase vector uses the "sandwich approach": here, the foreign antigen is flanked at its N-terminal side by parts of the bacteriophage λ *cI* (25-28), *cII* (29), or *cro* proteins (30-33) or by parts of the *E. coli* *OmpF* protein (34) and at its C-terminal side by an amino-terminally deleted version of β-galactosidase. The vectors have unique cloning sites between the two prokaryotic protein moities, which allows insertion of foreign DNA. If an open reading frame (ORF) exists in the inserted fragment and if this ORF is in register with the reading frames of both flanking procaryotic genes, a tripartite fusion protein will be expressed. Examples of di- and tripartite fusion proteins—in this case expressing immunogenic moities of the glycoprotein D (gD) of herpes simplex virus type I (HSV 1)—is shown in Figure 1.

It often was observed that the fusion of bacterial protein moities to both the N-terminal and C-terminal sites of an unstable foreign antigen resulted in the formation of a stable fusion protein. In several of these vectors (25, 28,29,34), the two translational reading frames of the proteins are not in

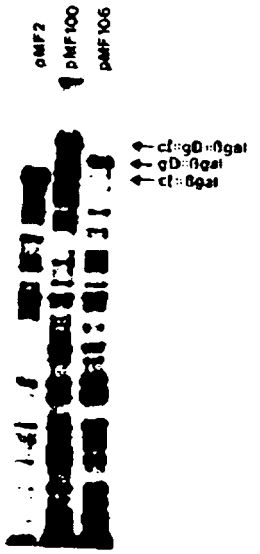

Figure 1 Autoradiogram of fusion proteins expressing immunogenic moities of gD of HSV-1. The figure shows a gel with (35S)L-methionine-labeled proteins in the *E. coli* K12 strain D27A1 carrying the indicated expression plasmids. Arrows indicate the positions of fusion proteins. (From Ref. 42).

register (frameshift mutation) and therefore give rise to a relatively low level of β-galactosidase activity. The insertion of foreign DNA at the cloning site(s) can reverse the frameshift mutation and thus results in the production of relatively high levels of β-galactosidase activity. This activity can be used to identify the respective recombinant plasmids in a *lacZ*-negative bacterial host. The production of a high level of β-galactosidase activity and a large fusion protein guarantees the cloning of a DNA fragment with at least one ORF that traverses the entirety of the fragment.

This method is particularly useful for "shotgun" cloning approaches and can identify, clone, and express open reading frame DNA from among a large collection of DNA fragments. Employing one of the above ORF vectors to express the hepatitis B virus X product, it was possible to detect in some HBV-positive sera from patients diagnosed as suffering from chronic active hepatitis antibodies that specifically recognized the hybrid protein (35). It was concluded that the X ORF encodes a protein and that this protein is antigenic in man.

Properties of β-Galactosidase Fusion Proteins

High-level expression of all three types of β-galactosidase fusion proteins often results in the formation of protein aggregates, also called "refractile

bodies" or "inclusion bodies." In most cases, the foreign antigen can be obtained in sufficient yield as a fusion protein, even if it cannot be expressed sufficiently in its mature, unfused form. There are reports, however, that indicate that the site of fusion can effect the stability and yield of a particular antigen. Stanley, for example, observed (33) that insertion of DNA fragments encoding parts of the E1 protein of Semliki Forest Virus (SFV) close to the 5' end of the *lacZ* gene gave rise to hybrid proteins, which were rapidly degraded. Insertion of the same fragments at the 3' end (into the EcoRI site) of the *lacZ* gene, however, resulted in the synthesis of stable hybrid proteins, which precipitated in an insoluble form within the bacteria. The author speculated that N-terminal addition of foreign polypeptides results in some perturbation of the β-galactosidase structure such that it becomes a substrate for endogenous proteases, whereas C-terminal addition guarantees that the bulk of the β-galactosidase polypeptide is translated before expression of the insert DNA, thereby establishing a normal pattern of polypeptide folding. As the size of the E1-encoding fragment increased, however, the level of hybrid protein expressed decreased, indicating that even the C-terminal fusion approach does not always guarantee a stable expression of the foreign antigen.

A similar finding was reported by Amann et al. (27) for the glycoprotein C (gC) of HSV 1. The cloning of gC-encoding DNA fragments into pUC vectors did not result in the expression of significant amounts of gC-related polypeptides. After insertion of the same fragments into a fusion vector, however, significant amounts of cI::gC::β-galactosidase fusion proteins were synthesized. These tripartite fusion proteins were immunologically reactive with anti-HSV 1 sera. In these experiments it also was observed that the hybrid proteins were present in different abundancies in the *E. coli* extracts and that their stability decreased as the size of the gC fragment increased.

Other Fusion Vectors

Other widely used fusion vectors include those that employ a prokaryotic fusion partner gene product of the *E. coli trp* operon (*trp*D, *trp*E, or a *trp*LE fusion) (13-15,36-39), of the *E. coli* β-lactamase (9-11), of the replicase of bacteriophage MS2 (12,40,41), or of the bacteriophage λ *cI* (42), *cII* (29,43-47), and *cro* (48) proteins. Other vectors use the protein A of *Staphylococcus aureus* as the fusion partner (49). Some of the protein A vectors are designed for the expression of intracellular protein A fusion proteins, others for the secretion of such fusion proteins from *E. coli*. The latter system, however, is limited to the efficient secretion of small polypeptides only fused to protein A, but may be used for the generation of antibodies against short peptides (50). The protein A fusion can be easily recovered from the culture medium of *E. coli* by a one-step procedure using IgG affinity chromatography.

Recently, prokaryotic fusion partners are expanded to include the glutathione-S-transferase (GST) of *Schistosoma japonicum* (51). In the majority of cases, these fusion proteins are soluble in aqueous solutions and can be purified from crude bacterial lysates under nondenaturing conditions by affinity chromatography on immobilized glutathione. The vectors have been engineered so that the GST carrier can be cleaved from the fusion proteins by digestion with site-specific proteases, following which the carrier and any uncleaved fusion protein can be removed by absorption on glutathione-agarose. A similar approach uses the maltose-binding protein (MBP), the product of the *E. coli malE* gene as the fusion partner and maltose-affinity chromatography for the purification of the resulting fusion protein (52).

As with these two vector system, a one-step purification method utilizing affinity chromatography of soluble hybrid proteins that have β-galactosidase activity have been described (53). The purified hybrid proteins can be used to obtain antibodies against the foreign portion of the protein fusion. The strongly and selectively expressed major capsid protein of bacteriophage T7 has also been recently utilized as the fusion partner in a new series of plasmid vectors (54).

In the case of intracellular high-level fusion protein expression, the resulting fusion protein will in most cases be insoluble and can be extracted by standard procedures developed for inclusion body purification (55-57). In order to ease the identification and purification of hybrid proteins, monoclonal antibodies against the prokaryotic fusion partner have been developed, for example, against the cII protein of bacteriophage λ (47) and against the *E. coli* β-galactosidase (58).

IDENTIFYING IMMUNORELEVANT ANTIGENS OF COMPLEX PATHOGENS

One particularly powerful method has been described in which expression libraries are constructed and screened with antisera (59,60). In this method, foreign DNA (genomic or cDNA) is inserted into the EcoRI site of the *lacZ* gene in a bacteriophage λ vector (λgt11), promoting synthesis of hybrid proteins. Efficient screening of antigen-producing clones in λgt11 recombinant libraries is achieved through lysogeny of the phage library in "high-frequency lysogeny" (hflA) mutant cells of *E. coli*. Upon induction, lysogens produce detectable quantities of cloned antigens and can be screened at high cell density by appropriate antisera. Critical in this method is, of course, the quality of the available antiserum. Human immune or hyperimmune sera, pooled or from individual donors, polyclonal or monospecific experimental animal sera, and monoclonal antibodies can be used for the cloning of immunorelevant antigens of the pathogen (for detailed technical protocols to use the λgt11 system, see Refs. 61 and 62).

This method has been successfully used to identify antigens of a variety of viral, bacterial, and parasitic pathogens, many of which are now tested as immune-stimulating components of vaccines. By using human immunesera, for example, λgt11-*Plasmodium falciparum* expression libraries have been successfully screened to identify immunodominant antigens of the human malaria pathogen *P. falciparum* (63,64).

In a similar shotgun-cloning approach of randomly generated fragments of *P. falciparum* genomic DNA employing a N-terminal β-galactosidase fusion vector (21,22) screening with human african immunesera detected a novel blood stage antigenic determinant characterized by degenerated repeats of a peptide nonamer (65). In another case, a λgt11-*P. falciparum* expression library has been probed with a polyclonal rabbit antiserum raised against the affinity-purified major blood stage p190 surface antigen (66). Parts of the respective cDNA prepared from total poly(A)$^+$ RNA of blood stage *P. falciparum* isolate could be cloned. One λgt-11 clone, which reacted strongly in the immunoenzymatic screening protocol, included the 3′ end of the p190 mRNA sequence encoding the final 70 amino acids at the C-terminus of the protein. At least this part of the p190 antigen, thus, must contain one or more immunogenic determinants.

λ-gt11 clones expressing p190 antigenic determinants were also used to develop a technique called "antibody select." In this technique, hybrid protein is bound to nitrocellulose and used to affinity-purify antibodies from the original probe. The selected antibodies are monospecific and can be used to confirm or identify any foreign gene sequences expressed in bacteria. It also provides a way to purify from complex serum antibodies against defined regions of a single antigen molecule (66).

The screening of λgt11 expression libraries with monoclonal antibodies has been particularly useful to precisely localize genes on large genomes. This technique has been used, for example, to locate the gene encoding a family of antigenically related DNA-binding proteins on the 240-kb human cytomegalovirus (CMV) genome (67). In order to map this gene, a random library of CMV DNA was generated in λgt11. The library was screened with a mixture of monoclonal antibodies directed against the "infected cell protein 36" (ICP36), and reactive clones could be isolated. The ICP36-coding sequence could subsequently be precisely mapped on the viral genome by using DNA from immunoreactive λgt11 clones as hybridization probe.

Mouse monoclonal antibodies have also been successfully utilized to identify and clone the five most immunogenic *Mycobacterium leprae* protein antigens with molecular weights of 65, 36, 28, 18, and 12 kDa (68,69) from λgt11 libraries. Later it was shown that the 65-kDa antigen carries important B-cell epitopes (70) and the 18-kDa antigen major T-cell epitopes (71) (see below).

DECIPHERING IMMUNODOMINANT EPITOPES ON CLONED ANTIGENS

Antigenic determinants, or epitopes, are specific segments of antigens recognized by antibodies or T cells. Approaches used to determine the precise location and/or amino acid sequence of protein epitopes include sequence analysis of viral variants that are resistant to neutralization by antibodies, screening purified proteolytic fragments, and screening collections of overlapping synthetic fragments (see Refs. 72,73). The application of recombinant DNA techniques in combination with monoclonal antibodies to this problem has resulted in new strategies to map antigenic determinants. The shotgun cloning of DNA into λgt11 and related fectors, for example, followed by screening with monoclonal antibodies results in the identification of antigenic determinants rather than in the cloning of the entire protein-coding sequence. Several options exist after the isolation of such recombinants: if the cloned DNA fragment is small in size, its sequence information can be used to design and synthesize peptides, which are subsequently coupled to carrier proteins that can be used to determine their antibody inducing and protective potential. Alternatively, the DNA fragments encoding such epitopes can be excised from the vectors (in case of the λgt11 vector with *Eco*RI) and subsequently utilized to clone and sequence the complete gene using standard techniques. Subsequently, a sub-library containing fragments of the gene with random endpoints is constructed in the λgt11 vector. The expression of epitope-coding sequences by individual recombinant bacteriophages is detected with monoclonal antibody probes, and the appropriate DNA clones are isolated. The precise nucleotide sequences of the cloned DNA fragments are determined by using primer-directed DNA sequence analysis. The DNA sequence encoding the epitope is attributed to sequences that are shared by multiple antibody-positive clones.

Employing mouse monoclonal antibodies, this technique was applied, for example, to the identification of six different epitopes of the major immunologically relevant 65-kDa protein of *M. leprae* (70). Each of the six epitopes investigated was determined to lie within 13-35 amino acids. One of the antigenic determinants elucidated with this approach is unique to *M. leprae*, and the remainder are shared with the 65-kDa proteins from a number of other mycobacteria.

T lymphocytes play a critical role in most aspects of the immune defense against disease (74). Because genes for the major protein antigens of *M. leprae* as seen by mouse monoclonal antibodies have been identified (68,69), it has become possible to test whether these individual antigens are recognized by T cells. Mustafa et al. (71) have screened crude λgt11 phage lysates of *E. coli* containing individual *M. leprae* antigens using *M. leprae*-specific T-cell clones

isolated from *M. leprae*-vaccinated volunteers. Using this method, the authors found that nearly half of the *M. leprae*-specific T-cell clones are stimulated to proliferate by lysates containing an epitope of a *M. leprae* protein of 18 kDa. This experiment shows that human T-cell clones recognize an epitope, expressed in *E. coli* as a β-galactosidase fusion protein, on a major *M. leprae* protein antigen. This method is generally applicable to the identification of parasite polypeptide antigens recognized by T cells.

Nunberg et al. (75) have used a virus neutralizing monoclonal antibody to precisely map the corresponding neutralizing antigenic determinant on the feline leukemia virus envelope protein gp70. In their method, short DNase I-generated DNA fragments encoding portions of the protein of interest are cloned into the EcoRI site of β-galactosidase in a bacteriophage λ vector to obtain expression of random protein fragments as fusion proteins. DNA of immunoreactive phage can be analyzed and sequenced rapidly to determine the neutralizing antigenic determinant. This procedure was used to map the neutralizing epitope of gp70 to a 14-amino-acid region in the amino-terminal half of the protein.

In a different approach, several defined regions of the phosphorylated matrix protein pp150 of human CMV were expressed as β-galactosidase fusion proteins, and these were tested for their immunoreactivity with human sera and their immunogenicity (76). One antigenic region could be expressed in large amounts and was found to carry immunodominant epitopes as shown by immunoblotting and ELISA.

Once the antigenic determinants have been localized using one of these alternative approaches, the encoding DNA fragments can be subcloned into plasmid expression vectors (described above) in order to maximize their expression levels. An alternative approach is to utilize the sequence information for the design of synthetic peptides that mimic the antigen determinants and couple them to a large carrier, such as KLH (keyhole limpet hemocyanin), or albumin. In general, the advantage of large fusion proteins is that they are immunogenic without being coupled to a carrier, in contrast to small proteins. In some recent approaches, B-cell epitopes have been combined with a T-cell epitope on the same synthetic peptide, an approach also applicable to fusion proteins. The purified fusion proteins or coupled synthetic peptides can subsequently be used for immunization and challenge experiments.

PROTECTIVE POTENTIAL OF HETEROLOGOUS ANTIGENS IN ANIMAL MODEL SYSTEMS

A potential limitation of the fusion protein approach to define epitopes is the difficulty presented by the class of antibodies that recognize topographically complex protein determinants. Protein epitopes can be divided into

two structural classes (see Ref. 73). A *segmental site* occurs within a continuous segment of the polypeptide. An *assembled topographic site* consists of amino acid residues located far apart in the primary sequence but brought together in the surface topography of the native protein through folding. For several proteins, it has been estimated that at least one-third of monoclonal and polyclonal antibodies made by immunizing with native protein react with assembled topographic sites (73). These assembled determinants may not form the appropriate structure outside the protein's native environment. For this reason, the fusion protein approach is not likely to be useful for mapping assembled topographic sites. With these estimates it is quite surprising that fusion proteins (which often form protein aggregates in *E. coli* have to be solubilized using SDS, urea, or guanidinium-hydrochloride, resulting in the denaturation of the fusion protein) are recognized by antibodies and still can be immunogenic.

Active immunization of mice with a HSV 1 envelope protein (gD-1Leu53-Ala312 antigen) expressed in *E. coli* as a fusion protein, for example, showed that the animals were protected from a lethal challenge with HSV 1 and HSV 2 (77,78) (Table 2). Moreover, antisera from rabbits immunized with the same gD-1 antigen also conferred passive immunity to mice against a challenge infection with either HSV 1 or HSV 2 (Fig. 2). On the other hand, various gC-1 and gC-2 fusion proteins from *E. coli* failed to induce protective immunity. The sera from immunized mice were not able to react with the authentic, glycosylated gC-1 and gC-2 envelope proteins, whereas sera raised against authentic gC-1 and gC-2 glycoproteins do recognize the gC fusion proteins from *E. coli* These results indicated that *E. coli* might represent an ideal host for expressing gD antigens as a possible component of a HSV vaccine, whereas gC antigen cannot be produced in an immunocompetent form in *E. coli*. Most likely, the protective antigenic determinant present on the gD-1 antigen will assume its natural conformation after renaturation of the gD-1 fusion protein and therefore constitutes a segmental site. In the case of the gC-1 and gC-2 envelope glycoproteins the protective antigenic site is either an assembled topographic site which does not assume its natural conformation after expression in *E. coli* or alternatively the antibodies induced after immunization of the unglycosylated antigen from *E. coli* are unable to bind and neutralize gC epitopes on intact viruses or infected cells due to interference of sugar chains of this highly glycosylated protein.

In a different example, a cDNA coding for 165 amino acids of the histidine alanine-rich protein HRPII of *P. falciparum* was isolated (40). The coding region was expressed in *E. coli* as a MS2-polymerase fusion protein, which was purified, solubilized in urea, and used for immunization of *Aotus* monkeys. The animals immunized with this fusion protein showed only low parasitemias (< 2%) after infection with *P. falciparum*, while animals from the

Table 2 Protection Experiments with Recombinant gD-1

Expt. no	pBD21-encoded fusion protein µg/immunization	Adjuvant	Immunization at day prior to challenge			Protection rate[a] after challenge with HSV-1	Protection rate[a] after challenge with HSV-2
1	200	$Al(OH)_3$	-42	-28	-14	7/10	
	200	PICLC	-42	-28	-14	9/10	
	67	PICLC	-42	-28	-14	10/10	
	20	PICLC	-42	-28	-14	9/10	
	200	PICLC		-28	-14	10/10	
	200	PICLC			-14	7/10	
	100	PICLC		-28	-14	10/10	
	25	PICLC		-28	-14	7/10	
	5	PICLC		-28	-14	7/10	
	1,5	PICLC		-28	-14	1/10	
3	100	-		-28	-14	9/10	
	25	-		-28	-14	6/10	
	6	-		-28	-14	4/10	
	1,5	-		-28	-14	2/10	
4	25	-		-28	-14	7/10	
	25	PICLC		-28	-14	9/10	
	25	$Al(OH)_3$		-28	-14	10/10	
5	100	-		-28	-14		8/10
	100	PICLC		-28	-14		10/10
	25	-		-28	-14		7/10
	25	PICLC		-28	-14		9/10
	6	-		-28	-14		3/10
	6	PICLC		-28	-14		8/10
6	100	-			-14	0/15	0/15
	100	PICLC			-14	1/15	7/15
	100	$Al(OH)_3$			-14	0/15	12/15
	25	-			-14	0/15	5/15
	25	PICLC			-14	0/15	11/15
	6	-			-14	1/15	5/15
	6	PICLC			-14	0/15	10/15
7	100	-			-14		2/10
	100	$Al(OH)_3$			-14		6/10
	100	CFA			-14		9/10
	100	IFA			-14		7/10
	100	PICLC			-14		5/10
	100	MPL + TDM			-14		6/10
	100	Zinc aspartate			-14		7/10
	100	Zinc lactate			-14		7/10

[a] Survivors per group

PICLC: Polyriboinosinic-polyribocytidylic acid complexed with carboxymethylcellulose; CFA: complete Freund's adjuvant; IFA: incomplete Freund's adjuvant

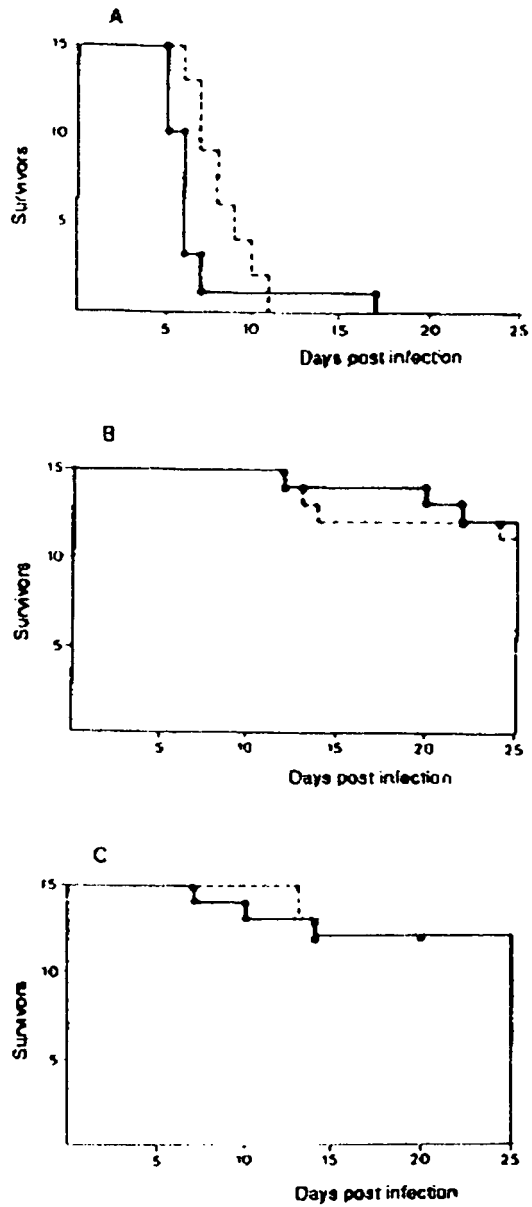

Figure 2 Protection of mice against a lethal challenge with HSV 1 (black circles) and HSV 2 (open circles) after administration of a rabbit immunoglobulin preparation to human IgA with an anti-HSV ELISA titer of < 1/40 (A), an IgG preparation (Beriglobin) with a titer of 1/80,000 (B), and a rabbit immunoglobulin preparation to recombinant gD-1 encoded by pBD21 with a titer of 1/4000 (C). (From Ref. 78.)

control group or animals immunized with a MS2-fusion protein carrying other malaria-specific sequences were not protected. This result suggests that this antigen is a good candidate for a malaria vaccine and demonstrates that fusion proteins are capable of conferring protection in challenge experiments.

FOREIGN ANTIGENS IN THEIR NATIVE CONFORMATION AT THE SURFACE OF *SALMONELLA* VACCINE STRAINS

Expression of foreign genes in *E. coli* at high rates has used the technique of fusion proteins in order to secure initiation from authentic prokaryotic signals and to form cytoplasmic inclusion bodies, which results in protection against bacterial proteases. In an alternative approach, protection can also be achieved via the export of foreign proteins through fusion with membrane proteins or proteins secreted from the bacteria. Major outer membrane proteins reach very high concentrations in enterobacteria and also are quite stable due to very low turnover rates. Employing outer membrane or fimbrial proteins for construction of fusion proteins has been successful but so far limited in the size of foreign polypeptides accepted in these carrier systems (see Chapter 5). One such system employing flagella from *Salmonella* and *E. coli* was used for the expression of a cholera toxin epitope (79), for HBsAg epitopes (80), and for epitopes of the *P. berghei* circumsporozoite antigen (81).

The flagella expression system, however, is only able to present single epitopes (approximately 20 amino acids) of well-defined antigens. Insertion of larger fragments is likely to disrupt the structural organization of the flagella and therefore does not result in efficient expression. Another approach for the surface expression uses the lamB protein of *E. coli*, which allows for the insertion of up to approximately 60 amino acids (82) and has successfully been used to induce antibodies against the VP1 protein from polio virus I (83,84) and against the pre-S antigen of HBV (84-86).

In a different approach, the outer membrane protein A (OmpA) has been used as a fusion partner for the surface expression of foreign antigens (87, 88). While several other outer membrane proteins such as OmpC, OmpF, and LamB are known to form oligomers and to form pores of limited specificity, the OmpA protein is found inserted into the membrane as a monomer. No dominant physiological function is known for this protein since OmpA mutants stay viable. As for other proteins of this class, OmpA is, however, known to be specifically recognized by a number of bacteriophages and colicins as an external receptor. This property has been used to detect segments of the polypeptide chain located at the exterior bacterial surface through sequence analysis of phage-resistant mutants. Four regions of 12-14 residues each have been defined in this way, which together constitute a regularly

spaced pattern in the N-terminal half of the protein. Based on these observations and further structural models, Pistor and Hobom (87,88) have constructed OmpA expression vectors which allow in-frame sandwich fusions of foreign genes. Oligonucleotides with suitable cloning sites were inserted in the *ompA* gene corresponding to positions of the third or fourth exterior surface domain of the mature protein. Following the initial insertion of various smaller segments of foreign polypeptides including 32 amino acids of antigenic determinant A of influenza HA, the authors succeeded to express an almost complete HA (514 residues) in an OmpA tripartite fusion. It could be shown that the HA is expressed after induction at the exterior surface of living *E. coli* cells. After transformation of the OmpA::HA expression plasmids into an attenuated *Salmonella typhimurium* strain and oral immunization with these live recombinant *Salmonella* of Balb/c mice, partial protection from an influenza virus challenge infection was observed (88).

After oral ingestion, *Salmonella typhimurium* enters deep tissues by attaching, invading, and proliferating in cells of the gut-associated lymphoid tissue (GALT). Delivery of an antigen to the GALT elicits generalized secretory, humoral, and cellular immune response. Therefore, avirulent *Salmonella* mutants that have lost the ability to cause disease without impairment of their ability to invade the GALT should be suitable to serve as effective vehicles to deliver foreign antibodies to the GALT to induce immunity. Construction of avirulent and immunogenic *S. typhimurium* strains has been accomplished successfully in several laboratories (see Chapter 12).

The *P. falciparum* blood stage antigens SERP and HRPII were recently cloned (40,41), and the latter one, expressed as a fusion protein with the MS2 polymerase and used for immunization of *Aotus* monkeys, induced protective immunity in these animals (40). Employing the OmpA vectors developed by Pistor and Hobom, the immunogenic moieties of these malarial antigens have been expressed in *E. coli* and in an attenuated *Salmonella typhimurium* vaccine strain (89,90). Figure 3 depicts the structure of expression plasmids which induce the synthesis of the respective SERP and HRPII fusion proteins. Upon induction, the malaria-specific sequences of 189 (HRPII) and 451 (SERP) amino acids, fused into the OmpA protein at one of the exposed domains, have been expressed. By indirect immunofluorescence studies, live bacteria expressing the fusion proteins react specifically with anti-SERP and anti-HRPII sera, respectively, indicating that the hybrid OmpA proteins become integrated into the bacterial outer membrane and expose the malarial antigens at the exterior surface. Moreover, immunogold staining experiments and trypsin treatment of live *E. coli* cells expressing the HRP::OmpA and SERP::OmpA fusion proteins confirmed the surface exposition of these malarial antigens. Mice which were immunized orally with *S. typhimurium* cells expressing HRPII and SERP on their surface show a humoral immune

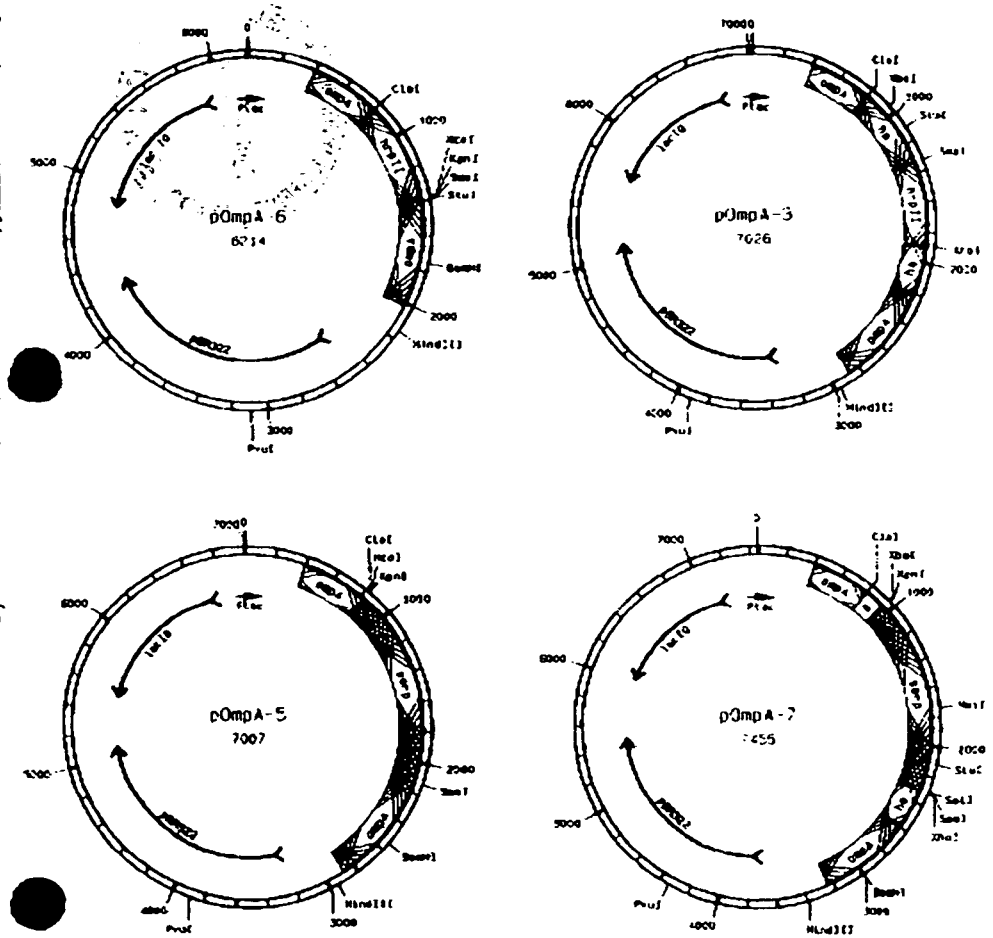

Figure 3 Structure of OmpA fusion plasmids expressing SERP and HRPII antigens. Coding region and sizes of OmpA, HA, HRPII, and SERP fused genes are shown. Relevant restriction sites, in particular those at gene fusion junctions, are indicated. (From Ref. 89.)

response as determined by the anti-SERP and anti-HRPII IgG and IgM titers (Fig. 4). From these and the above HA experiments it can be concluded that the OmpA surface expression system in combination with established *Salmonella* vaccine strains can be used to efficiently deliver large antigens to the mucosal system. Moreover, all experiments so far indicated that the foreign antigens assume their natural conformation on the surface of the recombinant bacteria.

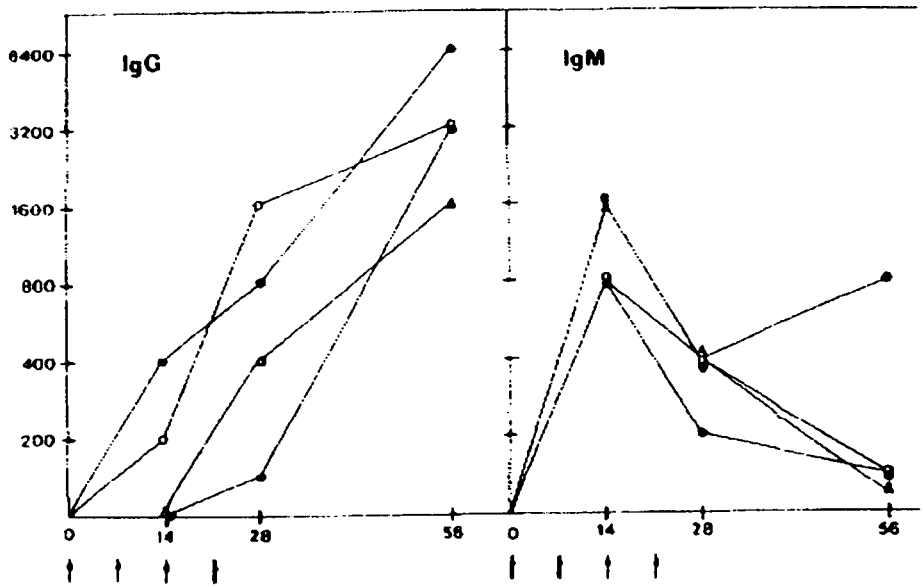

Figure 4 Antibody response of mice immunized orally with *S. typhimurium* strain SR-11 expressing HRPII and SERP OmpA fusion proteins on its surface. Mice were orally immunized at days 0, 7, 14, 21 (indicated by arrows). Blood samples were taken at days 0, 14, 28, and 56, pooled, and tested for the presence of HRPII and SERP specific antibodies by ELISA. For Ig typing, anti-IgG and anti-IgM specific POD conjugated goat antibodies were used. Asterisks = pOmpA-3; open circles = pOmpA-6; triangles = pOmpA-5; filled circles = pOmpA-7. The titer refers to the highest dilution of test serum at which the ratio of A492 of test serum to A492 of preimmune serum was >2.0. (From Ref. 89.)

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